Course Code : BPHT 3003 Course Name: Pharmaceutical Microbiology

Biochemical Test of Bacteria

Disclaimer

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CARDOHYDRATE FERMENTATION TEST

(Sugar Fermentation Test)

Aim: To determine the ability of microbes to ferment carbohydrates with the production of an acid and/or gas.

Principle:

Sugars are metabolized through different metabolic pathways depending on types of microbial species and aerobic or anaerobic environment .If fermenting bacteria are grown in a liquid culture medium containing the carbohydrate, they may produce organic acids as by-products of the fermentation. These acids are released into the medium and so lower pH of medium. If a pH indicator such as phenol red or bromocresol blue is included in the medium, the acid production will change the medium from its original color to yellow.

Gases produced during the fermentation process can be detected by using a small, inverted tube, called a Durham tube, within the liquid culture medium. If gas is produced, the liquid medium inside the Durham tube will be replaced; by the gas in the form of a bubble.

Interpretation:

If the medium changes from colorless to yellow and gas bubble is found in Durham's tube then it indicates acid and gas production. In some cases gas may not be evolved during the process. If no change observed in the colour of medium then sugar is not degraded by the organism.

CARBOHYDRATE FERMENTATION TEST (DURHAM TUBES)

Nutrient Broth + Respective Sugar From left to right: uninoculated positive, and negative.

INDOLE PRODUCTION TEST

Aim: To determine the ability of microbe to degrade the amino acid tryptophan.

Principle:

Tryptophan —(Tryptophanase)→ Indole + Pyruvic acid + Ammonia

P – dimethylaminobenzaldehyde + Indole —(HCl, alcohol; Dehydration reaction)→ Quinoidal red – violet compound

Interpretation:

Development of cherry red colour at the interface of the reagent and the broth, within seconds after adding the Kovacs' reagent indicates the presence of indole and the test is positive. If no colour change is observed, then the test is negative and so organisms are not capable of producing tryptophanase.

INDOLE TEST

TRYPTONE BROTH

Tube on the left is positive (E. coli); tube on the right is negative.

METHYL RED TEST (MR TEST)

Aim: To differentiate *E.coli* and *E.aerogen* and to determine the ability of microbes to oxidize glucose with production and stabilization of high content of acid end product.

Principle: ***E.coli:***

Glucose + H_2O → Lactic acid, Acetic acid, Formic acid + Methyl Red → CO_2 + H_2 + Red colour (positive test) (pH 4)

E. aerogen:

Glucose + H_2O → Acetic acid → 2,3butanediol + CO_2 + H_2O + Methyl Red → Yellow colour (negative test) (pH 6)

METHYL REDTEST

MR – VPbroth

Tube on left is positive (E. coli); tube on right is negative.

VOGES – PROSKAUER TEST (VP TEST)

Aim: To differentiate the *E.coli* and *E.aerogen* by the production of 2,3 – butanediol and acetoin via glucose fermentation.

Principle:

This test determines the capability of some organisms to produce non-acidic or neutral end products, such as acetyl methyl corbinol (acetoin), from the organic acid that results from glucose metabolism. This test is characterizes *E.aerogen*. Test identifies bacteria that ferment glucose and leading to 2,3-butanediol accumulation in the medium.

Glucose + ½ O_2 → 2 Pyruvate → α- aceto acetate CO_2 → Acetoin → 2,3-butanediol CO_2

CO_2

Acetoin + α- napthol —(40% KOH, Absolute alcohol)→ Diacetyl + Creatine (pink coloured complex)

Interpretation:

Development of crimson red colour indicates positive test for *E.aerogen*.

And no colour change indicates negative test.

VOGES-PROSKAUER TEST

MR – VPbroth

Tube on left is positive (E. aerogenes); tube on right is negative.

CITRATE UTILIZATION TEST

Aim: To determine the ability of the microbes to ferment citrate as sole carbon source.

Principle:

- Citrate as a sole carbon source for their energy needs.
- Presence of a citrate permease that facilitates transport of citrate into the bacterium.
- Sodium citrate as the carbon source, $NH4^+$ as a nitrogen source.
- pH indicator - bromothymol blue.
- This test is done on slants since O_2 is necessary for citrate utilization.
- When bacteria oxidize citrate, they remove it from the medium and liberate CO_2.
- CO_2 combines with sodium (supplied by sodium citrate) and water to form sodium carbonate - an alkaline product.
- This raises the pH, turns the pH indicator to a blue color, and represents a positive citrate test; absence of a color change is a negative citrate test.
- Citrate-negative cultures will also show no growth in the medium and the medium remains green.

Sodium citrate $\xrightarrow[\text{Citrase}]{\text{Citrate permease}}$ Pyruvic acid + Oxalacetic acid + CO_2

Excess sodium from Sodium Citrate + CO2 + H_2O $\longrightarrow$ Na_2CO_3 (pH $\downarrow$) (green $\rightarrow$ blue)

CITRATE UTILIZATION TEST

Simmon Citrate agar

Left to right: uninoculated, positive (E. aerogenes), and negative.

NITRATE REDUCTION TEST

Aim: To determine the ability of some microbes to reduce nitrate (NO_3^-) to nitrites (NO_2^-) or beyond the nitrite stage.

Principle:

- Certain organisms like Chemolithoautotrophic bacteria and many chemoorganoheterotrophs can use nitrate (NO_3^-) as a terminal electron acceptor during anaerobicrespiration.
- In this process, nitrate is reduced to nitrite (NO_2^-) by nitrate reductase.
- Further reduce the nitrite to either the ammonium ion or molecular nitrogen.
- Nitrate broth medium containing 0.5% potassium nitrate (KNO_3).
- Examined for the presence of gas and nitrite ions in the medium.

$$NO_3^- + 2H^+ + 2e^- \xrightarrow{\text{Nitrate reductase}} NO_2^- + H_2O \xrightarrow{\text{Other enzymes}} NH_3 + \rightarrow \tfrac{1}{2} N_2$$

Sulfanilic acid + α –naphthylamine + Nitrite ions (Colourless) → Sulfobenzene azo – α-naphthylamine (red coloured)

Nitrate Reduction Test

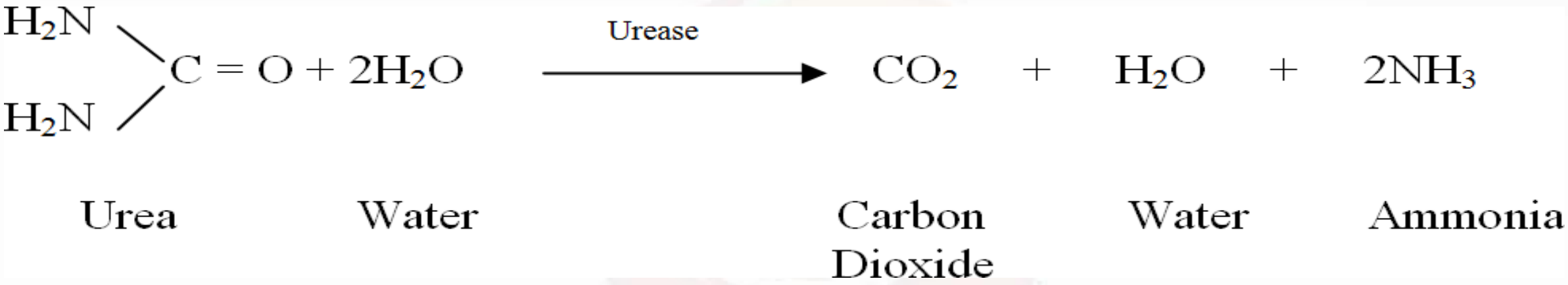
Peptone nitrate broth on left is positive (*E. coli); tube on right is negative.*

UREASE TEST

Aim: To determine the ability of microbes to degrade urea by urease.

Principle:

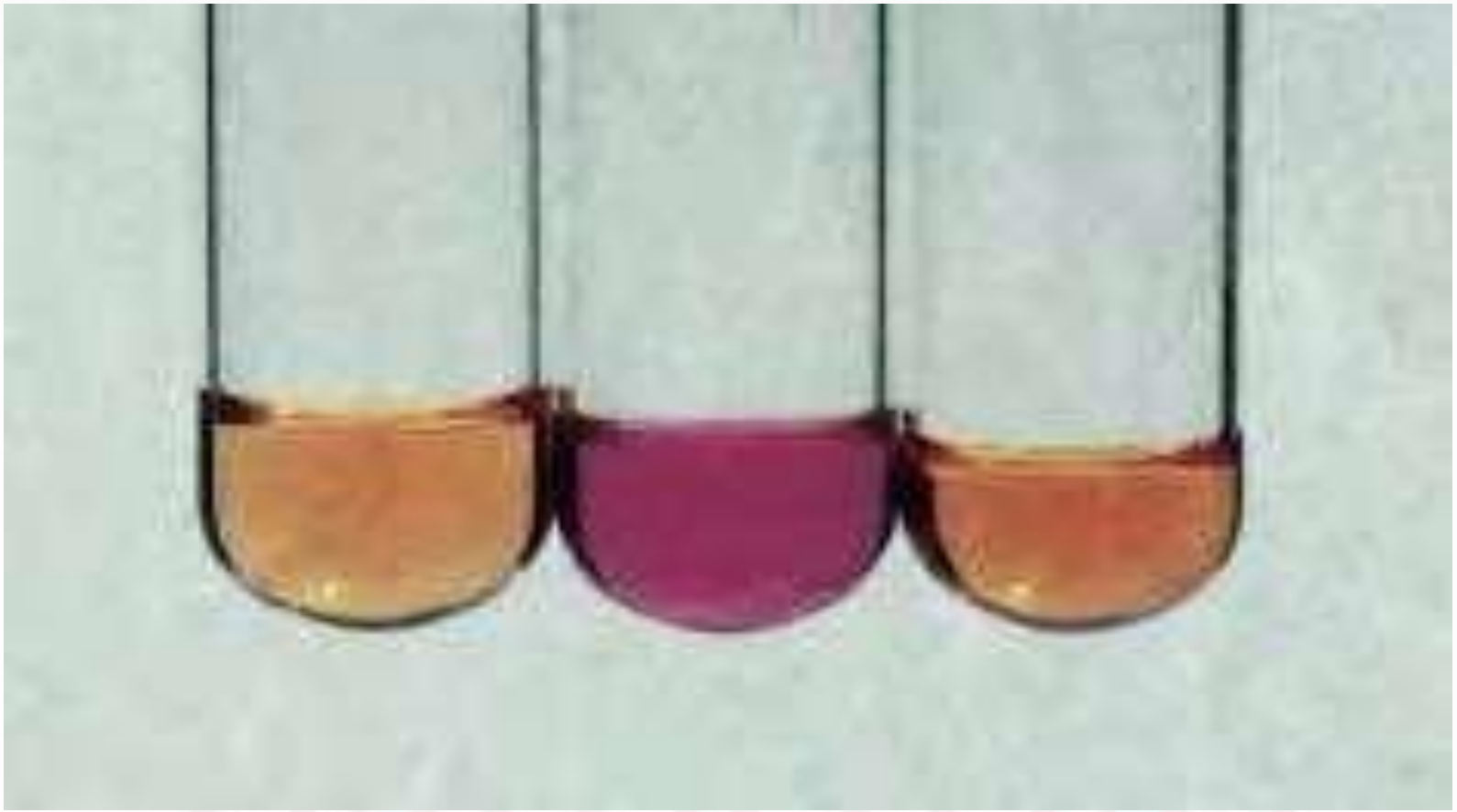
- Urea is diamide carbonic acid often referred as carbamide.
- The hydrolysis of urea is catalysed by specific enzyme urease to yield 2 moles of ammonia.
- Urease attacks the nitrogen and carbon bond in urea and forms ammonia.
- The presence of urease is detected, when the organisms are grown in urea broth.
- Medium containing the pH indicator phenol red.
- Splitting of urea creates the alkaline condition which turns phenol red to deep pink in colour.
- Mainly used for identification of *Proteus spp*. from other genus of lactose nonfermenting enteric organisms.



Interpretation:

If urea is present in the medium, then it will be degraded which creates alkaline condition in the medium which result in colour change from reddish pink to deep pink.

UREASE TEST



UREABROTH
From left to right—uninoculated, positive
(*Proteus) and negative*

TSI (TRIPLE SUGAR IRON) AGAR TEST

Aim: To differentiate among and between the members of *Enterobacteraceae family.*

Principle:

•Study and identify the organisms belonging to Enterobacteraceae family.

•It is also used to distinguish the *Enterobacteriaceae* from other gram- negative intestinal bacilli (by their ability to catabolize glucose, lactose, or sucrose, and to liberate sulfides from ferrous ammonium sulfate or sodium thiosulfate.)

• TSI agar slants contain a 1% concentration of lactose and sucrose, and 0.1% glucose.

•The pH indicator, phenol red, is also incorporated into the medium to detect acid production from carbohydrate fermentation.

• The uninoculated medium is red in colour due to presence of phenol red dye.

•Yeast extract, beef extract and peptone provides nitrogen, sulphur, trace elements and vitamin B complex etc.

• NaCl maintains osmotic equilibrium.

•Lactose, Sucrose and Dextrose are the fermentable carbohydrates.

•Sodium thiosulfate and ferrous sulfate make H_2S indicator system.

•Thiosulfate is reduced to H_2S by several species of bacteria and H_2S combines with and form insoluble black precipitates. $FeSO_4$ present in the medium

• Blackening usually occurs in butt of tube.

•Incubation is for 18 to 24 hours in order to detect the presence of sugar fermentation, gas production, and H2S production.

The indicator is pink at alkaline pH and yellow at acidic pH, at neutral pH it remains red.

The following reactions may occur in the TSI tube:

- **Yellow butt (A) and red slant (K)** due to the fermentation of glucose (phenol red indicator turns yellow due to the persisting acid formation in the butt). The **slant remains** red (alkaline) (K) because of the limited glucose in the medium and, therefore, limited acid formation, which does not persist.
- A **yellow butt (A) and slant (A)** due to the fermentation of lactose and/or sucrose (yellow slant and butt due to the high concentration of these sugars) leading to excessive acid formation in the entire medium.
- **Gas formation** noted by splitting of the agar.
- **Gas formation (H_2S)** seen by blackening of the agar.
- **Red butt (K) and slant (K)** indicates that none of the sugars were fermented and neither gas nor H_2S were produced.

Interpretation:

- Alkaline slant / acidic butt ⟶ only glucose is fermented.
- Acidic slant / acidic butt ⟶ glucose, sucrose and lactose all 3 sugars are fermented.
- Bubbles or crack present ⟶ gas production.
- Black precipitate present ⟶ H_2S production.

TSI AGAR TEST

	Tube a	Tube b	Tube c	Tube d
Slant	–	A	K	K
Butt	–	A	K	A
Gas	–	+	–	–
H_2S	–	–	+	+

OXIDASE TEST

Aim: To determine the ability of microbes to produce Oxidase enzyme

Principle:

- Oxidase enzyme plays a key role in Electron Transport Chain during aerobic respiration.
- Cytochrome Oxidase catalyzes the oxidation of reduced Cytochrome by molecular oxygen (O_2), resulting in the formation of H_2O and H_2O_2.
- Aerobic as well as some facultative anaerobes and microaerophillic bacteria shows oxidase activity.

IN CELL:

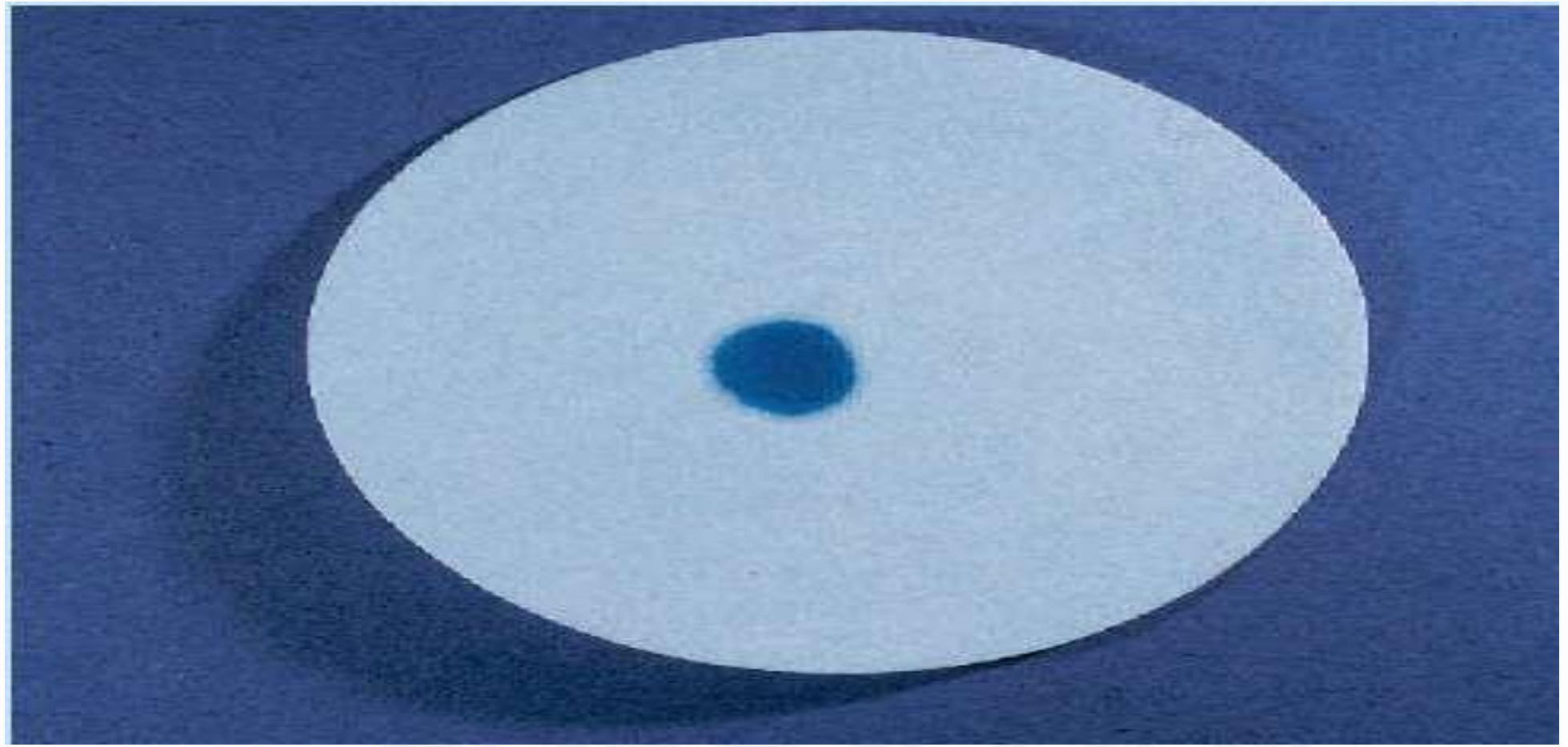
2 reduced Cytochrome C + 2 H^+ + ½ O_2 $\xrightarrow{\text{Cytochrome Oxidase}}$ 2 oxidized Cytochrome C

Purple

IN TUBE:

2 oxidized Cytochrome C + *p* – aminodimethylalanine oxalate ⟶ 2 reduced Cytochrome C + Wurster's Blue (dark purple)

Oxidase Test



Note the purple to dark purple color after the colonies have been added to filter paper moistened with oxidase reagent.

CATALASE TEST

Aim: To determine the ability of an organism to produce catalase.

Principle:

- Certain organisms produce hydrogen peroxide during aerobic respiration and sometimes extremely toxic superoxide radicals.
- These are extremely toxic because they are powerful oxidizing agents and destroy cellular constituents very rapidly.
- A bacterium must be able to protect itself against such O_2 products or it will be killed.
- Many bacteria possess enzymes that afford protection against toxic O_2 products.

•Obligate aerobes and facultative anaerobes usually contain the enzymes superoxide dismutase, which catalyzes the destruction of superoxide

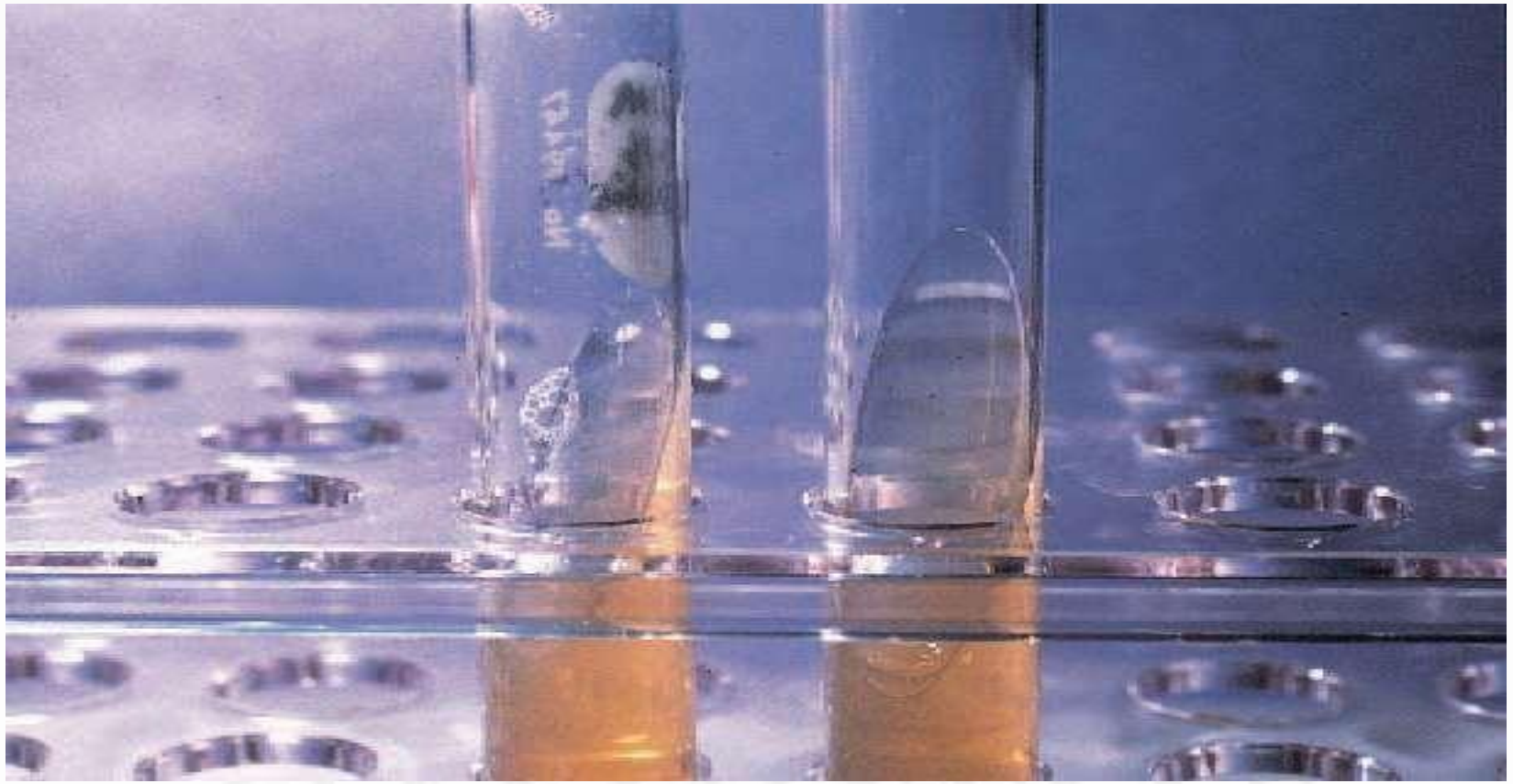
•And either catalase or peroxidase, catalyze the destruction of hydrogen peroxide.

•Catalase production and activity can be detected by adding the substrate H_2O_2 to an appropriately incubated (18- to 24- hour) tryptic soy agar slant culture.

•If catalase was produced by the bacteria, they will liberate free O_2 gas on reaction.

•Bubbles of O_2 represent a positive catalase test; the absence of bubble formation is a negative catalase test.

Catalase Test on Slants



Tryptic soy agar slants

(a) Staphylococcus aureus,
catalase positive. Notice the bubbles of oxygen (tube on theleft).

(b) Enterococcus faecalis,
catalase negative; note the absence of bubbles (tube on theright).

STARCH HYDROLYSIS

Principles:

✓Many bacteria produce enzymes called **hydrolases.**

✓Hydrolases catalyze the splitting of organic molecules into smaller molecules in the presence of water.

✓The starch molecule consists of two constituents:

- **Amylose, an unbranched glucose polymer (200 to 300** units)
- **Amylopectin, a large branched polymer.**

✓Both amylopectin and amylose are rapidly hydrolyzed by certain bacteria,

✓ Using their **α-amylases, to yield** dextrins, maltose, and glucose.

Interpretation:

✓Gram's iodine can be used to indicate the presence of starch.

✓ When it contacts starch, it forms a blue to brown complex.

✓ Hydrolyzed starch does not produce a colour change.

✓If a clear area appears after adding Gram's iodine to a medium containing starch and bacterial growth:

- ➢Amylase has been produced by the bacteria.

✓If there is no clearing, starch has not been hydrolyzed.

LIPID HYDROLYSIS

Learning Objectives

1.Understand the biochemical process of lipid hydrolysis

2.Determine the ability of bacteria to hydrolyze lipids by producing specific lipases

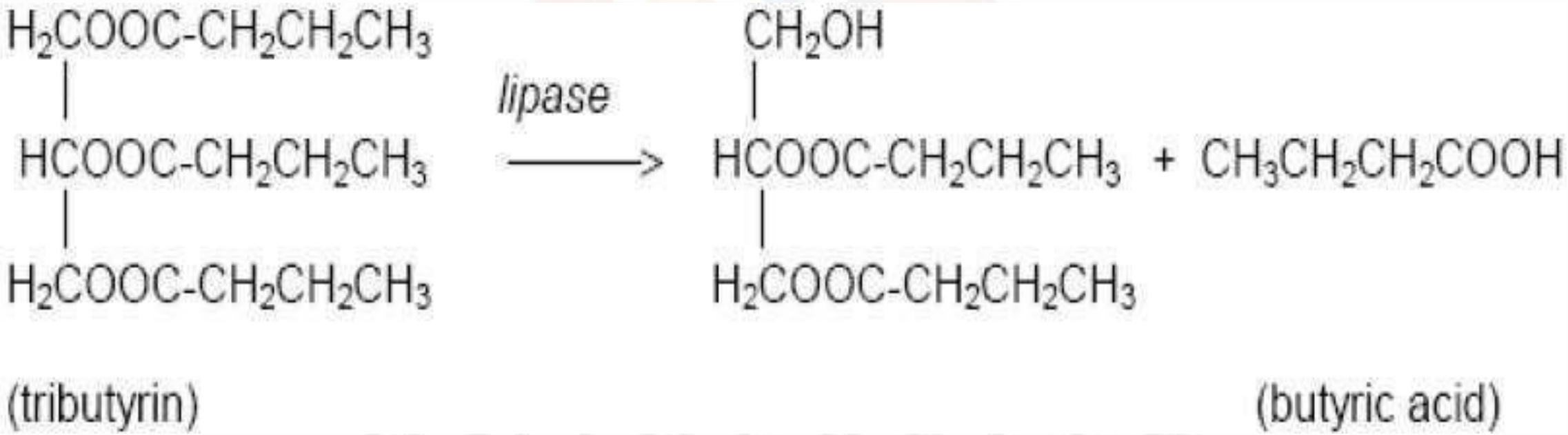
3. Perform a lipid hydrolysis test

Principles:

- ✓Lipids are high molecular weight compounds possessing large amounts of stored energy.
- ✓The two common lipids catabolized by bacteria are the **triglycerides (triacylglycerols) and phospholipids.**
- ✓Triglycerides are hydrolyzed by the enzymes called **lipases into glycerol and free fatty acid molecules**.
- ✓Glycerol and free fatty acid molecules can then be taken up by the bacterial cell and further metabolized through reactions of :
  - ✓glycolysis, β-oxidation pathway, and the citric acid cycle.
  - ✓These lipids can also enter other metabolic pathways where they are used for the synthesis of cell membrane phospholipids.
- ✓Since phospholipids are functional components of all cells, the ability of bacteria to hydrolyze host-cell phospholipids is an important factor in the spread of pathogenic bacteria.

✓In addition, when lipase-producing bacteria contaminate food products.,

✓The lipolytic bacteria hydrolyze the lipids, causing spoilage termed **rancidity.**



(tributyrin) (butyric acid)

✓The culture medium contains tributyrin as a reactant; degradation of this compound gives rise to clear zones surrounding the lipolytic colonies in the otherwise turbid culture medium.

Reference:

- James. G, Cappuccino, Natalie Sherman, Microbiology. A Laboratory Manual, 4th edition. Pg.No : 129 – 175.
- John P. Harley, Lansing M. Prescott, Laboratory exercises in Microbiology, 5th edition. Pg.No. : 125 – 175.
- Patel. R.J; Patel.K.R; Experimental Microbiology, volume – I, Aditya Publication. Pg.No : 110 – 127.
- Web resources

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